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Alterations in the cellular genome affecting the expression or function of genes controlling cell growth or differentiation are the mechanism underlying the genesis of all cancers. One avenue of cancer research studies the function of normal growth-control genes (proto-oncogenes), as well as their transformation into cancer genes (oncogenes). A subset of proto-oncogenes comprise the RAS signal transduction pathway.

Vulval development in the nematode worm *Caenorhabditis elegans* is controlled by a RAS signal transduction pathway. *C. elegans* has proven to be a powerful model system for understanding regulation of the RAS pathway. This research studies two novel components in this RAS pathway. The *sur-2* gene, which encodes a large, novel protein, was identified by a screen for suppressors of activated RAS. Using a yeast two-hybrid screen, I have identified a novel protein which binds to SUR-2. My analysis of this protein is presented. I have also identified a second novel gene in the RAS suppressor screen, called *sur-9*. My phenotypic analysis, epistasis analysis, mapping and cloning of *sur-9* are presented. These studies will improve our understanding of mitogenic signaling and may eventually lead to the design of novel cancer therapeutic agents which target control points in the RAS pathway.

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Abstract

Characterization of two ras-signaling components in *C. elegans*

Alterations in the cellular genome affecting the expression or function of genes controlling cell growth or differentiation are the mechanism underlying the genesis of all cancers. One avenue of cancer research studies the function of normal growth-control genes (proto-oncogenes), as well as their transformation into cancer genes (oncogenes). Ultimately, it is hoped that this basic research will yield novel cancer therapies that target these oncogenes. A subset of proto-oncogenes comprise the RAS signal transduction pathway, a highly conserved collection of signaling molecules used by all eucaryotes. This pathway is of special significance in that it is used by many mitogenic signaling cascades, and mutations in RAS and other components of this pathway are found in many human tumors. This research uses a simple model system to better understand the RAS signaling pathway.

The vulva is the egg-laying and mating organ in the nematode worm *Caenorhabditis elegans*. Formation of the vulva is induced by activation of a RAS signal transduction pathway. This research characterizes two novel components within this pathway. This information will improve our understanding of mitogenic (growth control) signaling and may eventually lead to the design of novel cancer therapeutic agents which target control points in the RAS pathway. Previous studies have identified components within the *C. elegans* RAS pathway by using genetic screens for suppressors of an activated RAS multivulval (Muv) phenotype. These screens have defined RAF, MEK and MAP kinase as intermediates in the pathway. Two of the novel genes identified in these screens are studied in this work.

One of the novel genes identified in these screens is *sur-2* (Genes & Dev. (1995) 9;2251-2265). Worms homozygous for *sur-2* loss-of-function alleles show the vulvaless (Vul) phenotype. Epistasis analysis suggests that SUR-2 acts downstream or in parallel to *sur*-1/MAP kinase. The SUR-2 gene product bears no significant homology to known signaling proteins; hence, its biochemical activity remains unknown. It is my hope that identifying SUR-2 interacting proteins in a yeast two-hybrid interactor screen may yield clues to SUR-2 function or regulation. Using a SUR-2 bait construct in the screen, I have identified a novel *C. elegans* gene product that interacts with SUR-2. I am currently pursuing analysis of this novel cDNA, and my analysis is presented here.

A second gene, *sur-9*, was also identified by the ability of a mutant allele to suppress the Muv phenotype caused by a RAS gain-of-function mutation. The *sur-9* mutation alone produces a worm capable of VPC induction, albeit with vulval and other developmental defects. Linkage analysis places the *sur-9* gene on chromosome III, and fine mapping has localized the *sur-9* gene within a 0.47 map unit domain. Epistasis analysis shows that this gene acts late in the RAS pathway. Attempts to clone the *sur-9* gene are currently underway. My analysis of *sur-9* is presented.

Background/Significance

RAS-signaling is a highly conserved pathway

RAS-mediated signal transduction is a pathway utilized by all eucaryotes to regulate basic cellular functions. This pathway transduces diverse physiological signals in multiple tissues and at all stages of development. Although a variety of extracellular signals are received through specific transmembrane receptors, many of the subsequent cellular responses appear to be mediated by a cascade of common intermediates, within which RAS is a key component (reviewed in Ahn, 1993). The signaling activity of the RAS protein is modulated by its bound guanine nucleotide, as well as the ancillary proteins SOS (an exchange factor, aka guanine nucleotide releasing factor), GAP (a RAS-GTPase activating protein), and adapter proteins (e.g. GRB2). Following receptor and RAS activation, RAS recruits and activates the serine/threonine kinase RAF at the cell membrane through a poorly understood mechanism. Activated RAF then triggers a kinase cascade which includes MEK (a dual-specificity kinase, aka MAP kinase kinase) and ultimately MAP kinase, another serine/threonine kinase. Following its phosphorylation and activation, MAP kinase is translocated to the nucleus, where it is thought to control programs of gene expression by phosphorylating target transcription factors (reviewed in Hill and Treisman, 1995; Karin, 1994; Treisman, 1994). Despite our reasonably complete knowledge of this pathway, an underlying question still remains. Since a variety of extracellular signals utilize this same intracellular cascade, there must be other aspects to regulation of the pathway or targets of the MAP kinase which confer the specificity of a cellular response.

Ras pathway intermediates are frequently abrogated in breast cancer

Alterations in the cellular genome which affect the expression or function of genes controlling cell growth or differentiation are considered to be the underlying cause of all cancers. One avenue of current research in cancer biology endeavors to identify and understand the function of normal growth-control genes (proto-oncogenes), as well as understand their transformation into cancer genes (oncogenes). Ultimately, it is hoped that basic research into the nature of proto-oncogenes and oncogenes will yield cancer therapies that target these genes. A subset of proto-oncogenes comprise the RAS signal transduction pathway, a highly conserved collection of signaling molecules used by all eucaryotes.

The RAS-mediated signal transduction pathway includes growth factors, growth factor receptor tyrosine kinases, RAS proteins, other signaling protein kinases, nuclear proteins, and transcription factors. These signal transducing molecules play a vital role in regulating cell proliferation and differentiation. It is these two processes that are disrupted in cancers and give rise to the destructive neoplastic phenotype. Breast cancer, specifically, shows deregulation of a number of signal transduction components. The following is a partial list of genes (and their respective proteins) that have been theorized to play a role in breast cancer

progression or offer important prognosticators for response to therapy: RAS (Bos, 1989; El-Ashry and Lippman, 1994), EGF receptor/c-*erbB*1 (El-Ashry and Lippman, 1994; Klijn et al., 1993; LeMaistre et al., 1994), EGF receptor-related oncogenic receptors *erbB*-2 (aka HER2/*neu*), *erbB*-3, *erbB*-4 (El-Ashry and Lippman, 1994; Janes et al., 1994; LeMaistre et al., 1994), CSF-1 and CSF-1 receptor (Kacinski, 1995), Grb2 (Daly et al., 1994), EGF and TGF-α (Klijn et al., 1993), estrogen and progesterone receptors (El-Ashry and Lippman, 1994), and insulin-like growth factors (IGF-I and IGF-2), their receptors, and IGF binding proteins (IGFBPs) (Klijn et al., 1993; Yee, 1994).

C. elegans vulval differentiation entails multiple signaling events.

The vulva is the mating and egg-laying organ in the adult nematode worm. The structure arises from relatively few precursor cells, is formed from simple cell lineages, and is readily observed under the dissecting microscope. The presence or absence of this structure provides a sensitive and easily assayed phenotype for the study of the vulval development program. Prior to vulval induction, six vulval precursor cells (VPCs), numbered P3.p thru P8.p, have equal developmental potential (see Figure 1). Each has the ability to assume a vulval fate or become part of the surrounding hypodermis. During the third larval stage, three of the six VPCs (P5.p-P7.p) differentiate into one of two vulval cell types (1° or 2° fates). The 1° and 2° cells are distinct in both the lineages they produce and their morphologies. The remaining three VPCs become part of the surrounding hypodermis (3° fate).

The fate assumed by each of the VPCs is determined by three different intercellular signaling events (see Figure 1). These signals are: (1) an inhibitory signal emanating from the hypodermis to repress the vulval 1° and 2° cell fates; (2) an inductive signal from a specialized cell residing in the adjacent gonad called the anchor cell (this inductive signal induces three of the six VPCs to assume 1° or 2° vulval cell fates); (3) a lateral signal acting between VPCs to specify 2° cell fate, called lateral inhibition (reviewed in Horvitz and Sternberg, 1991).

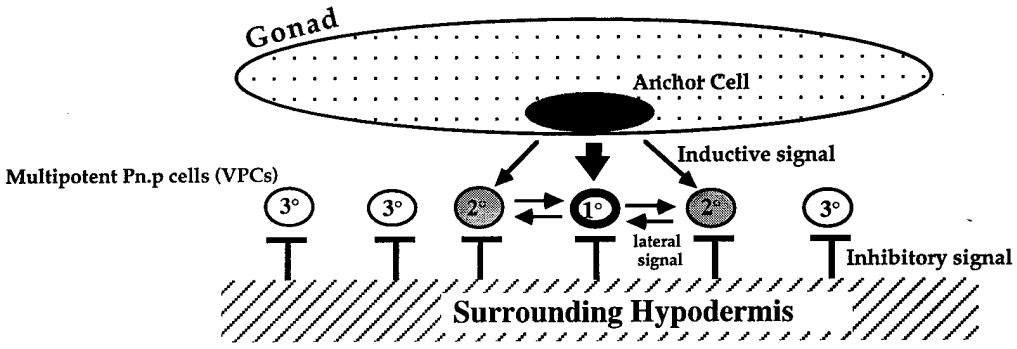

Figure 1. Schematic of vulval precursor cell (VPC) signaling. VPCs P3.p thru P8.p are shown, as well as the 1°, 2°, or 3° fates they will eventually assume. The following signaling events are indicated: (1) an inhibitory signal from the surrounding hypodermis; (2) an inductive signal emanating from the anchor cell within the gonad; (3) a lateral signal between the VPCs.

Mutations that result in mis-specification of VPC fates have defined many genes required for normal VPC differentiation. In these mutants, one of two phenotypes may be manifested (see Figure 2). There may be more than three VPCs that assume a vulval fate (1° or 2° cell fates), resulting in extra vulval tissue in the mature worm (the multivulva or "Muv" phenotype). Alternatively, greater than three VPCs may assume a hypodermal cell lineage (3° fate), resulting in a vulvaless (or "Vul") phenotype (Ferguson and Horvitz, 1985; Ferguson and Horvitz, 1989; Ferguson et al., 1987; Horvitz and Sulston, 1980, reviewed in Horvitz and Sternberg, 1991).

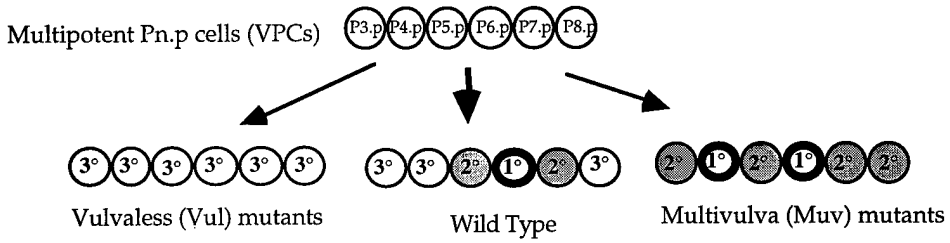

Figure 2. Schematic of possible VPC developmental fates and the resulting phenotypes. VPCs P3.p thru P8.p are shown, along with the 1°, 2°, or 3° fates they may assume. Wild type, vulvaless, and multivulva phenotypes are represented.

Ras-mediated signal transduction components are utilized in the *C. elegans* vulval induction signaling cascade.

The invertebrates *Drosophila melanogaster* and *Saccharomyces cerevisiae* have been fruitful model systems in the study of RAS-mediated signal transduction (reviewed in Herskowitz, 1995; Zipursky and Rubin, 1994). The nematode worm *C. elegans* has also been a rich source for study. A large number of genes that control

nematode vulval cell lineages have been described. A subset of these genes comprise a RAS signal transduction pathway controlling vulval development (Ferguson and Horvitz, 1985; Ferguson and Horvitz, 1989; Ferguson et al., 1987; Horvitz and Sulston, 1980, reviewed extensively in Eisenmann and Kim, 1994; Han and Sundaram, 1996; Horvitz and Sternberg, 1991; Kayne and Sternberg, 1995; Kornfeld, 1997; Sternberg, 1993; Sternberg and Han, 1998; Sundaram and Han, 1996). Most of the signal transduction intermediates in this *C. elegans* signaling cascade have counterparts in mammals. Specifically, the inductive signal emanating from the anchor cell in the *C. elegans* gonad to induce 1° and 2° vulval lineages in the VPCs requires the same genes found in mammalian mitogenic signaling systems (see Figure 3).

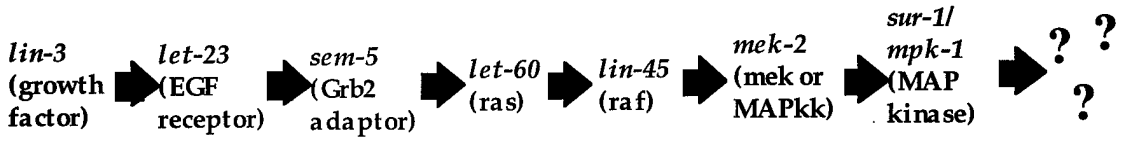

Figure 3. Schematic of *C. elegans* vulval signaling pathway. *C. elegans* gene nomenclature is shown, with the mammalian homologs shown in parenthesis. References: *lin-3* (Hill and Sternberg, 1992), *let-23* (Aroian et al., 1990), *sem-5* (Clark et al., 1992), *let-60* (Han and Sternberg, 1990), *lin-45* (Han et al., 1993), *mek-2* (Kornfeld et al., 1995; Wu et al., 1995), *sur-1/mpk-1* (Lackner et al., 1994; Wu and Han, 1994).

Furthermore, the identification of novel signaling molecules in *C. elegans* using genetic strategies has led to the identification of previously unknown mammalian homologues, including *sur-8* (Sieburth et al., 1998), *sur-5* (Gu et al., 1998) and *sur-6* (unpublished data, Sieburth and Han), and has led to better understanding of their mammalian counterparts, as in *ksr-1* (Kornfeld et al., 1995; Sundaram and Han, 1995; Therrien et al., 1995). Our work may ultimately permit the development of novel "molecular therapies" for the treatment of breast cancer which target control points in the RAS pathway (Brugge, 1993; Levitzki and Gazit, 1995). Our lab has also demonstrated the utility of *C. elegans* as a tool in cancer research by showing that *C. elegans* vulval development is a sensitive multicellular assay system to directly test the effectiveness of potential anticancer drugs that control activity of the RAS pathway (Hara and Han, 1995).

In an attempt to identify additional components which regulate this RAS signaling pathway (including signaling components downstream of MAP kinase), a genetic suppressor screen was used to identify genes which, when mutated, would suppress the Muv phenotype caused by a *let-60(n1046)/ras* gain-of-function mutant allele. The mutations identified in the screen would be anticipated to disrupt genes which lie within or regulate activity of the RAS signaling pathway. This screen obtained mutations in the *lin-45/raf, mek-2* and *sur-1*/MAP kinase genes, indicating that the screen was successful in identifying known RAS signal transduction intermediates (Lackner et al., 1994; Wu and Han, 1994; Wu et al., 1995). This research identifies and characterizes two novel genes also identified in these suppressor screens.

sur-2

One of the novel genes identified in the screen for genes that can suppress the RAS gain-of-function phenotype is called *sur-2*, for suppressor of activated ras (Singh and Han, 1995). Epistasis analysis verified that SUR-2 acts downstream of the *let-60*/RAS pathway and may act downstream of *sur-1*/MAP kinase. Worms homozygous for *sur-2* loss-of-function alleles are vulvaless, and show the bag-of-worms phenotype. *sur-2* mutant alleles demonstrate pleiotropic effects, implicating *sur-2* in a number of developmental events in the maturing worm. These effects include an incompletely penetrant larval lethality, males are unable to mate, possibly due to male tail developmental defects, and a variably penetrant sterility and gonadal defects. However, the genetic and molecular relationship of *sur-2* to other downstream components in the vulval signaling pathway, including *lin-1* (ETS family transcription factor), *lin-31* (winged-helix family transcription factor) and *lin-25* (novel) remain somewhat ambiguous.

The gene encodes a predicted 1,587 amino acid, 190-kD product bearing no significant homology to any known protein; hence, its biochemical activity remains unknown. The only recognizable protein motif within SUR-2 lies within the C-terminus, and contains repeating glutamine and histidine residues resembling *opa* repeats. *Opa* repeats are found in a wide variety of proteins, and may be sites of protein/protein interactions (Wharton et al., 1985). SUR-2 also contains a highly acidic domain reminiscent of transcription factor activation domains. The protein also contains a single consensus MAP kinase phosphorylation site as well as eight proline-directed serine/threonine phosphorylation sites. The experiments described in this research investigate the function and regulation of the *sur-2* gene and gene product in the vulval development program.

An exciting observation has recently been made by Arnold Berk at UCLA (personal communication). He has identified a human homologue of the nematode SUR-2 protein, and has evidence that the protein may be involved in adenovirus E1A-mediated transcriptional regulation in human cells.

This report describes my work to identify SUR-2 interacting proteins. Unfortunately, some of the initial experiments proposed have not been fruitful. These failed approaches included a SUR-2 genetic suppressor screen, production of anti-SUR-2 antibodies and heat-shock inducible expression of SUR-2. These negative results were discussed previously. Most significantly, I have recently identified a SUR-2 binding protein in the yeast two hybrid interaction screen, and this novel gene is currently being analyzed. That data is presented.

sur-9

A second gene, *sur-9*, was also identified by the ability of a mutant allele to suppress the Muv phenotype caused by a RAS gain-of-function allele. The *sur-9* mutation alone produces a worm capable of VPC induction, albeit with vulval and

other developmental defects. Mapping, cloning and preliminary characterization of this gene are described in this report.

Identification of SUR-2 interacting proteins

One goal in my project is to ascribe a biochemical function to the SUR-2 protein and determine how SUR-2 activity is regulated. I hope to make progress toward this goal by identifying the proteins which interact with SUR-2. I am currently testing known proteins that are candidates for interaction with SUR-2 by using the yeast two-hybrid interaction assay (Ausubel et al., 1990; Chien et al., 1991; Fields and Song, 1989). I have also screened two *C. elegans* libraries to identify SUR-2 interacting proteins. As with any result obtained in the yeast two-hybrid assay, observations I make will be confirmed by additional *in vitro* and *in vivo* experiments.

Testing candidate proteins for SUR-2 interaction

Methods/Results

I used the yeast two-hybrid assay to test candidate proteins which may physically interact with SUR-2. The SUR-2 bait construct consists of the first 1412 of the 1587 amino acids in the SUR-2 protein cloned into the yeast two-hybrid GAL4 DNA binding domain bait vector pAS2 (Clontech). All attempts to subclone the full length *sur-2* cDNA into the bait vector have been unsuccessful despite multiple subcloning strategies. The candidate cDNAs were subcloned into the two-hybrid GAL4 activation domain prey vector, pACT2 (Clontech). The yeast strains used in the screen contain two reporter genes, *HIS* and *lacZ*, which are activated upon reconstitution of the GAL4 DNA binding and transcriptional activation domains. The candidate proteins tested in the assay are listed below:

sur-1/MAP kinase -MAP kinase activities have been shown to have a wide variety of substrates, including transcription factors. *C. elegans sur-1*/MAP kinase had been shown to likely lie upstream or in parallel to *sur-2* by indirect epistasis evidence (Lackner et al., 1994; Singh and Han, 1995; Wu and Han, 1994). We are eager to test whether SUR-2 is a direct target of *C. elegans* MAP kinase activity. The *C. elegans* MAP kinase cDNA has been tested in the two hybrid assay with SUR-2. No interaction was seen.

lin-1 - The *lin-1* gene has been shown to be a negative regulator of the vulval induction pathway and acts downstream of MAP kinase in the pathway (Beitel et al., 1995). The *lin-1* cDNA encodes an ETS family transcription factor and contains MAP kinase consensus phosphorylation sites. A physical interaction

with *lin-31* which is regulated by MAP kinase has recently been described (Tan et al., 1998). The genetic relationship between *sur-2* and *lin-1* is unclear. A *lin-1* cDNA has been tested in the two-hybrid assay, and no interaction was detected.

lin-31 - The *lin-31* gene also lies downstream in the vulval induction pathway, and may lie downstream of MAP kinase. However, its phenotype is complex, and a null allele results in a randomization of VPC fates. The genetic relationship between *lin-31* and *sur-2* is unclear. The *lin-31* cDNA encodes an HNF-3/*fork head/winged-helix* family transcription factor and contains consensus MAP kinase phosphorylation sites (Miller et al., 1993). In addition, LIN-31 has been shown to physically interact with both LIN-1 and MAP kinase and is regulated by MAP kinase activity (Tan et al., 1998). A LIN-31 prey vector will be made and tested in the SUR-2 interaction assay.

lin-25 - The *lin-25* gene is a positive regulator of the vulval induction pathway, and was shown to act late in the induction pathway. The *lin-25* cDNA encodes a novel protein of unknown function (Tuck and Greenwald, 1995). Interestingly, it is observed that LIN-25 protein is downregulated in *sur-2* mutant worms (Nilsson et al., 1998). No interaction was detected with a *lin-25* cDNA in the two-hybrid assay.

lin-39 - The *lin-39* gene encodes a homeodomain transcription factor of the *Drosophila* Antennapedia/*sex combs reduced* class which controls a variety of cell fates in the central body region of the developing worm, including the fates of the vulval precursor cells (Clandinin et al., 1997; Maloof and Kenyon, 1998; Miller et al., 1996; Wang et al., 1993). Mutations in the *lin-39* gene result in vulvaless worms. Epistasis analysis demonstrates a dual role for *lin-39* both before and after the RAS/MAP kinase cascade. The LIN-39 signal may be required for VPCs to respond to the RAS/MAP kinase inductive signal. A LIN-39 prey vector is currently being tested in the SUR-2 interaction assay.

Library screening for SUR-2 interacting proteins

In addition to testing candidate proteins for interaction with SUR-2, I have also screened two *C. elegans* cDNA libraries for SUR-2 protein interactions. The libraries used in this screen are courtesy of Robert Barstead and are *C. elegans* mixed stage two-hybrid libraries, each cloned into the pACT2 GAL4 activation domain prey vector. One library, RB-1, is an oligo-dT primed library, while the second, RB-2, is a random primed library. Each of these libraries contains an estimated 1×10^7 unique clones. The yeast strains used in the screen contain two reporter genes, encoding *HIS* and *lacZ*.

Methods/Results

Using the RB-1 oligo-dT primed library, 0.24×10^7 clones were screened. No positives were identified using this library.

Using the RB-2 random primed library, 3.64×10^7 clones were screened. Of this number, 64 positive clones were initially characterized that activated both the *HIS* and *lacZ* reporter genes (see Table 1, p.18). Following this initial identification, the candidate clones were subjected to a series of control experiments. These controls included testing for the ability of the cDNA clone by itself to activate the reporter genes independent of the SUR-2 bait and also the ability to reconstitute the bait/prey interaction after isolating the bait and prey plasmids. The majority of clones failed these tests (51 of 65). The remaining 13 candidate positive clones were subjected to molecular analysis. Plasmid DNA was purified for each of the positive clones, and the cDNA inserts characterized by either PCR or restriction analysis. PCR product or purified plasmid was sequenced for each of these clones. It was determined that 7 of the 13 candidates contained *C. elegans* cDNAs that were subcloned in the antisense orientation, eliminating them as false positives. Of the remaining 6 candidate cDNAs in the sense orientation, three of these were cloned into incorrect reading frames. The remaining three candidates were identified as follows:

1) vitellogenin gene 2
2) vitellogenin gene 5
3) a novel gene product predicted by the *C. elegans* genome sequencing project

I chose to pursue analysis of the novel gene product. It appears that the 2.0 kB cDNA I identified in the screening is a full length clone, or near full length clone, since the sequence contains both 5′ and 3′ untranslated sequence. The *C. elegans* genome sequencing project placed the gene on cosmid B0379, which contains 40K of genomic sequence from *C. elegans* chromosome I. The sequencing project predicted this cosmid to contain five different open reading frame transcription units. However, the cDNA identified in my screen contained exons from two different predicted transcription units (genes B0379.4 and B0379.5), indicating the predicted gene structures on cosmid B0379 to be incorrect. Further analysis of the gene structure of my isolated clone is currently in progress.

Discussion/Conclusions

Of the candidate proteins so far tested for interaction with SUR-2, none have shown a positive result in the two-hybrid assay. It is feasible that none of these candidates form a physical association with SUR-2 *in vivo*, despite the fact that genetic analysis places them all to act late in the RAS/MAP kinase pathway. It as also possible that if these proteins do interact, the interaction may not be detected in the two hybrid assay, since activation or modifications of the proteins may be required to induce an interaction. Alternatively, steric hinderance caused by the

GAL4 fusion proteins may block the interactions, and may not be detected in the interaction assay.

More excitingly, the library screening has identified a candidate SUR-2 interacting protein. Although vitellogenin was also identified in the screen, it is not physiologically likely to act as a RAS signaling intermediate. Vitellogenin is an abundant egg yolk protein involved in development and its expression pattern in *C. elegans* is not consistent with action in the VPCs. In *C. elegans*, this protein is abundantly expressed in the larval stages, which might explain its over representation in our positive clone candidates. Of 29 clones sequenced, seven were vitellogenin genes. The vitellogenin genes that were sequenced were found in the sense and antisense orientations, and of those in the sense orientation, most were out of frame.

The B0379 gene is currently being analyzed for an *in vivo* role in vulval induction. As with any two-hybrid library screen result, positives must be verified by methods independent of the two-hybrid assay, preferably in a biological (*in vivo*) assay. I am currently analyzing gene B0379 by two physiologically-relevant means. First, I am using double-stranded RNA inhibition (RNAi) to test if removal of B0379 transcript from the developing worm has any effect on vulval induction or development. RNAi has been proven to be a valuable technique in studying loss-of-function phenotypes in genes for which no mutations are known (Fire et al., 1998; Montgomery et al., 1998). RNAi specifically phenocopies the phenotypes observed with genetic mutations. A second experiment I will undertake in the near future is to observe the expression pattern of the B0379 gene. To accomplish this, I will make a chimeric gene consisting of the B0379 promoter region linked to the green fluorescent protein (GFP) coding sequence. The resulting DNA construct will be used to make a transgenic worm, and the B0379 expression pattern will be observed in living worms.

Characterization and cloning of *sur-9*

The use of genetic screens has been a powerful tool in the elucidation of signaling pathway proteins in *C. elegans*. This has been especially true for understanding the RAS pathway. In the study of vulval induction, genetic screens in the worm have identified a EGF-like growth factor homologue, a receptor tyrosine kinase, RAS, RAF, MEK and MAP kinase within the RAS pathway. These genetic screens also identified genes which had no previously characterized mammalian homologues, including *sur-2, sur-5, sur-6, sur-8 and ksr-1*. All of these genes are now known to have mammalian homologues, making the worm an ideal model organism for the understanding of mammalian mitogenic signaling.

I have initiated the characterization and cloning of another gene identified by genetic criteria. One mutant allele of this gene was identified in a genetic suppressor screen by its ability to suppress the Muv phenotype caused by a *let-60(n1046)/ras* gain-of-function mutant allele.

Method/Results

Screening/chromosome mapping/complementation

A genetic suppressor screen was conducted to identify signaling components which lie downstream of RAS. A worm containing a *ras* gain-of-function mutant allele *let-60(n1046)* displays the multivulval (Muv) phenotype. In the screen, these Muv worms are exposed to the mutagen ethyl methanesulfonate (EMS), and after two generations, progeny which no longer show the Muv phenotype were isolated. This loss of the Muv phenotype is presumably due to acquisition of a secondary mutation (a suppressor mutation) elsewhere in the RAS pathway downstream of RAS which prevents expression of the Muv phenotype. One new suppressor mutation was linked to *C. elegans* chromosome III, and called *sur-9* (suppressor of activated ras), and given the mutant allele designation *sur-9(ku258)*. Quantitation of the suppression is given in Table 2 (p.19). Following the mapping to chromosome III, the mutant allele was subjected to allelic complementation tests using other suppressor gene alleles and previously characterized genes which also mapped to chromosome III. It was shown that the *sur-9* gene does not correspond to any previously identified gene on chromosome III.

Characterization of sur-9

Preliminary characterization of the *sur-9(ku258)* phenotype has been started (see Table 3, p.20). Three traits were quantitated: percentage of sterile animals, brood size, and percentage of animals that are unable to lay eggs (Egl). It is significant to note that not all *sur-9(ku258)* Egl animals are vulvaless. Some do have a vulva, but are unable to lay eggs, indicating some defect other than an inability to induce the vulval development pathway. Experiments currently in progress include lineage analysis of each of the VPCs in the *sur-9(ku258)* mutant.

sur-9 epistasis analysis

The position at which *sur-9* acts in the RAS pathway is a critical question in understanding how SUR-9 exerts its activity. I have initiated epistasis analysis using the *sur-9(ku258)* allele and other characterized components of the RAS pathway. Since *sur-9* has the ability to suppress the Muv phenotype of *let-60(n1046)/ras(gf)*, it is assumed that *sur-9* acts downstream of RAS in the pathway. The following genes were used in this epistasis analysis:

lin-15 - The LIN-15 gene products are thought to act as negative regulators of the *let-23* receptor tyrosine kinase (Clark et al., 1994; Huang et al., 1994). Their precise biochemical activity remains unknown. Loss of function alleles of *lin-15* display the Muv phenotype, similar to the *let-60(n1046)* phenotype. It might be predicted that worms containing both *lin-15* and *sur-9* mutations

would show the non-Muv phenotype similar to the *let-60(n1046);sur-9(ku258)* double mutant worms. I have made a *lin-15(n765-loss-of-function); sur-9(ku258)* double mutant worm. These worms show the non-Muv phenotype, as predicted.

lin-45/raf - RAF is a well characterized, but still not well understood, intermediate in the RAS pathway (Han et al., 1993; Sternberg et al., 1993). RAF acts in a positive manner, and is thought to be activated at the cell membrane. Naturally occurring *raf* gain-of-function alleles have not been identified in *C. elegans*. However, an artificial gene consisting of a Drosophila *raf* gain-of-function allele driven by a *C. elegans* heat shock promoter is able to induce a Muv phenotype in the worm when the gene is activated by heat shock. I have made a worm strain containing this *raf(gf)* construct and the *sur-9(ku258)* allele. I am currently testing whether the *sur-9* mutant allele is able to suppress the Muv phenotype caused by the *raf(gf)* heat shock construct.

lin-1 - As discussed above, the *lin-1* gene encodes an ETS family transcription factor that has been shown to be a negative regulator of the vulval induction pathway. The gene acts late in the RAS pathway, downstream of MAP kinase (Beitel et al., 1995; Tan et al., 1998). *lin-1* loss-of-function alleles result in a Muv phenotype. I have made double mutant worms containing *lin-1(e1275(lf));sur-9(ku258)*. These double mutant worms no longer show the Muv phenotype. This is a very significant observation, since this indicates that *sur-9* may act far downstream in the RAS pathway, and possibly downstream of MAP kinase.

sur-9 mapping and cloning

Three-point fine mapping was done to position the *sur-9* gene on chromosome III. Four rounds of mapping with increasingly finer genetic markers have localized the *sur-9* gene to a 0.47 map unit domain. This mapping used the marker pairs *unc-32* (0.00)/*dpy-18*(8.50), *dpy-18*(8.50)/*unc-64*(21.46), *dpy-19*(-0.16)/*unc-69*(2.32) and *unc-32*(0.00)/*emb-9*(0.47). The *sur-9* gene lies within the region bounded by *unc-32* and *emb-9*. The region is well represented by overlapping cosmids which completely cover the region. Combinations of 17 overlapping cosmids are currently being used in a standard microinjection cosmid rescue protocol to identify the cosmid containing the wildtype *sur-9* gene. Once a rescuing cosmid has been identified, the open reading frames on that cosmid will be subcloned to determine the identity of the *sur-9* gene.

Discussion/conclusions

Classical genetic screens have proven to be fruitful tools in the dissection of the RAS signaling pathway. It is hoped that the *sur-9* gene, identified for its ability

to suppress an activated RAS gene, will add to our understanding of how the RAS pathway is regulated. Once cloned, the *sur-9* gene and gene product will be studied. Future experiments will include expression pattern studies using GFP reporter constructs and analysis of the temporal requirements for *sur-9* expression using heat shock inducible expression vectors. If the *sur-9* gene sequence gives any clues as to its biochemical activity or placement in the RAS pathway, I will test if SUR-9 is able to physically interact with previously characterized components in the pathway.

Similar to the SUR-2 interacting gene B0379 described above, the *sur-9* gene may provide information on how the changes in gene transcription induced by the RAS pathway are able to program precursor cells to induce the vulval development pathway. This question of what comes after the activation of transcription factors by MAP kinase remains fundamental in understanding the RAS vulval induction pathway. Although the biochemistry of the pathway from the tyrosine kinase receptor through MAP kinase is reasonably well outlined, little is known of how the regulation of transcription factor activity determines vulval fates. Both the B0379 and *sur-9* genes may provide clues to this outstanding question.

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Table 1. Two-hybrid screen

	PCR Insert Size (kB)	XhoI Digest Insert size (kB)	Endogenous HIS3 and lac Z expression	Reconstitution of HIS3 and lac Z expression	Sequence/Blast Search	Sequence Orientation	Reading Frame
5/11 A-5	1.4	1.4	-	-	cystathion gamma-lysase	Sense	+3
5/11 B-11	1.7	1.4	-		malate synthase	Antisense	
5/14 A-3	1.6	1.9	-	-	C27C12.3, novel	Antisense	
5/19 A-3	2.8	2.4	-	-			
5/19 A-7	2.8	2.6	-	-	vittellogenin 4 or 5	Antisense	
5/19 A-41	2.2	1.8	-	-	vittellogenin 5	Sense	+1
5/19 A-44	2.1	1.9	-	-	M88.6, novel	Antisense	
5/19 A-118	1.9	1.0 +0.6	-	-	vittellogenin 1or 2	Antisense	
5/19 A-119	2.2	0.85 +0.7 +0.5	-	-	vittellogenin 5	Sense	+1
5/26 A-99	3.1 ?	3.2	-	-	collogen	Antisense	
5/26 A-100	1.2	0.8	-	+	EGF like domain	Sense	+3
5/26 A-128	1.1	0.9		+	vittellogenin 6	Sense	+3
5/26 A-133	2.3	2.2	-	-	K10D2.2, novel	Antisense	
6/8 #47	2	1.75	-	-	Zk945.3, novel	Antisense	
6/8 #55	0.7	0.6	-	+	serine carboxypeptidase	Antisense	
6/8 #70	2.8	2.5	-	-	ER ATPase	Antisense	
6/8 #87	2	1.75	-	+	B0379.5, novel	Sense	+1
6/8 #100	2	2	-	-	0		
6/15 A-60	2.3		-	+	Vitellogenin 5	Sense	result pending
6/15 A-95	1.7			-			
6/15 A-137	1.9 & 1.6	1.0 + .9	-	+	Novel	Antisense	
6/15 A-180	1.5		+	+			
6/22 A-34			-	-			
6/22 A-79	0?			-			
6/22 A-131			-	-			
6/29 A-4	2.3	2.2	-	+	Nucleoporin	Antisense	
6/29 A-12	~2.0-2.5	1.5+0.6	-	+	Proteosome Regulator	Antisense	
6/29 A-42	0?		-	-			
6/29 A-44	~1.5-2.0		-	-			
6/29 A-66		2.0+0.6			alpha actinin	Antisense	
6/29 B-1	~1.5-2.0		-	-			
6/29 B-7			+	-			
6/29 B-91			-	-			
6/29 B-93	~1.5-2.0		-	-			
6/29 B-129		2.0+0.5+0.3	-	+	Vitellogenin 2	Sense	+1
6/29 B-151-a			-	-			
7/7 A-75			-	-			
7/7 A-84			-	-			
7/7 A-103			-	-			
7/7 A-172			-	-			
7/7 B-22-a				-			
7/7 B-34			-	-			
7/7 B-54			+	+			
7/7 B-55			-	-			
7/7 B-63		3.8	-	+	Novel	Antisense	
7/7 B-70			-	-			
7/7 B-104			-	-			
7/16 A-7	1.7		-	+	Novel	Antisense	
7/16 A-8			-	-			
7/16 A-9			-	-			
7/16 A-12				-			
7/16 A-14			-	-			
7/16 A-22			-	-			
7/16 A-35-a	2.2		-	+	5' Nucleotidase	Sense	+2
7/16 B-21		1+0.3+0.2	-		Myosin Heavy Chain	Antisense	
7/16 B-22	2.3		-	+	Novel	Antisense	
7/16 B-29			-		EGF repeats protein	Sense	+2
7/16 B-44		1.6			novel	Antisense	
8/11 A-1				-			
8/11 A-3			-	-			
8/11 A-35			-	-			
8/11 B-20			-	-			
8/22 A-18			-	-			
8/22 A-25				-			
8/22 B-6			-	-			
8/22 B-83			-	-			

	let-60(n1046)/ras(gf) (n=146)	*let-60(n1046)/ras(gf); sur-9(ku258)* (n=251)	*sur-9(ku258)* (n=93)
Muv (%)	>79	1.6	0
non-Muv WT (%)	15	25.1	100
non-Muv Pvul (%)	<6	73.3	

Table 2. Suppression of *let-60(n1046)* Muv by *sur-9(ku258).*
Animals can display one of three phenotypes.
Multivulval, non-Muv with wildtype appearing vulva,
and non-Muv with protruding vulva (Pvul).

	Wildtype	*let-60(n1046)/ras(gf)*	*let-60(n1046)/ras(gf); sur-9(ku258)*	*sur-9(ku258)*
% sterile	0	<5	44	20
Brood size	~200	>150	20 (+/- SD 16.3)	62 (+/- SD 41.3)
% Egl	0	<1	29	28

Table 3. Characterization of *sur-9(ku258).*

Sterile animals produce no progeny. Brood size is the total number of viable eggs plus progeny that hatch within the mother (bag-of-worms). Egl animals are unable to lay eggs, although vulva may be present. Egl animals that were able to lay some eggs prior to showing the bad-of-worms phenotype were not included in the % Egl figure in the table.